

Article

Differences in Soil Inorganic Phosphorus Availability and *pqqC*-Harboring Bacterial Communities Between Long-Term Vineyards and Adjacent Uncultivated Soils

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Abstract

Soil phosphorus (P) availability is critical for vineyard productivity, and *pqqC*-harboring bacteria contribute to inorganic P (Pi) mobilization. However, knowledge of the coordinated responses of Pi partitioning and *pqqC*-harboring bacteria to prolonged grape cultivation remains limited. Here, soils from 20-year-old grape orchards (Y20) and adjacent uncultivated soils (CK) were compared to explore the relationships among Pi fractions, *pqqC*-harboring communities, and edaphic characteristics. Relative to CK, Y20 showed significantly lower pH and higher concentrations of labile and moderate labile Pi fractions. Although the *pqqC* gene abundance declined significantly in Y20, the Shannon diversity of *pqqC*-harboring communities increased, and community composition differed markedly between the two land-use types. Soil pH and labile Pi were the main factors shaping community composition. Network analysis revealed strong associations between *pqqC*-harboring taxa and soil P fractions, with *Azospira* serving as a key connector. Pearson correlation analysis further showed that labile and moderately labile Pi fractions were negatively correlated with soil pH and *pqqC* gene abundance, while NaHCO₃-Pi was positively correlated with the Shannon diversity. Overall, long-term grape cultivation redistributed soil Pi toward more bioavailable forms, closely associated with soil acidification and shifts in the abundance and diversity of *pqqC*-harboring communities.

Keywords: soil P availability; *pqqC* gene community; inorganic P mobilization; orchard; long-term cultivation



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1. Introduction

Grapevine is a perennial vine crop of high economic importance and is widely cultivated across the world owing to its strong adaptability to diverse environmental conditions [1,2]. Phosphorus (P) is an essential nutrient for crop growth and productivity, and it is involved in multiple physiological and metabolic processes in grapevines, such as energy transfer, photosynthesis, and nutrient acquisition. The P required by grapevines is mainly supplied by the soil [3,4]. However, despite its essential role, P availability frequently constrains agricultural production because soil phosphate generally has low solubility and readily combines with calcium, magnesium, iron, and aluminum to form sparingly soluble or insoluble compounds [5–7]. To satisfy crop P requirements and maintain vineyard

productivity, large quantities of chemical phosphate fertilizers derived from phosphate rock have been continuously applied to vineyard soils. Nevertheless, a considerable proportion of the applied P is converted into moderately labile inorganic P (Pi) and recalcitrant Pi fractions, resulting in the progressive accumulation of legacy Pi in soils [8]. Long-term excessive P inputs may gradually saturate the soil's capacity for Pi retention and adsorption, thereby increasing the likelihood of P loss from soils to adjacent water bodies [9,10]. Therefore, mobilizing and utilizing accumulated soil legacy Pi represents an important approach for maintaining the soil–plant P balance and improving P resource efficiency in orchard ecosystems. Soil phosphate-solubilizing bacteria (PSB) play a fundamental role in regulating the biogeochemical cycling of P and enhancing P availability to plants [6,11]. Among them, microorganisms carrying the *pqqC* gene are particularly important because they can increase soil P availability by solubilizing inorganic phosphate compounds [12,13]. The *pqqC* gene encodes PqqC, the enzyme that catalyzes the final step of pyrroloquinoline quinone (PQQ) biosynthesis. As a key component of the PQQ-dependent glucose oxidation pathway, PqqC contributes to gluconic acid production and thereby promotes the solubilization of mineral phosphates [14]. In perennial orchards, the structure and function of *pqqC*-harboring bacterial communities are strongly affected by long-term continuous monoculture, mainly through changes in soil physicochemical properties, including soil pH, inorganic orthophosphate, and soil organic carbon (SOC).

Soil acidification is one of the most common consequences of intensive and long-term agricultural management. Previous studies reported an average decrease of approximately 0.5 pH units in Chinese croplands from the early 1980s to the 2000s, largely due to excessive nitrogen (N) fertilization [15]. Compared with croplands, orchards in China often receive even greater nutrient inputs and thus face more severe soil acidification [16,17]. For example, a survey conducted in 2018 indicated that the average soil pH of pomelo orchards in southeastern China decreased by 0.8 units, from 5.1 to 4.3 [18]. In grape-growing systems, long-term cultivation for more than 10 years has also been reported to reduce soil pH and intensify soil acidification in the northeastern Jiaodong Peninsula [1,19]. Severe acidification has likewise been observed in citrus orchard soils, where the minimum recorded soil pH reached 3.2; moreover, soil pH was closely associated with orchard age, with longer cultivation periods corresponding to lower pH values [20]. A growing body of evidence indicates that long-term chemical fertilizer application can reduce the abundance of genes involved in P cycling, including those related to P dissolution, uptake, and transport, such as *pqqC*. It can also reshape P-acquiring microbial communities by modifying their diversity and taxonomic structure, largely as a result of soil acidification [21–24]. Tang et al. [13] further demonstrated that the abundance and diversity of P-cycling microorganisms, including *pqqC*-harboring microbes, change markedly when soil pH shifts from alkaline conditions (pH > 7.0) to acidic conditions (pH < 7.0). This indicates that the transition from alkaline to acidic soil may represent a critical threshold driving shifts in P-cycling microbial communities during soil acidification.

In addition to changing soil pH, acidification may also influence *pqqC*-harboring bacterial communities by altering C and P availability. Soil acidification has been shown to decrease labile C pools while increasing relatively stable C fractions, which may favor microorganisms with oligotrophic life-history strategies and thereby reshape *pqqC*-harboring bacterial assemblages [2,16,25]. Furthermore, acidification can increase soil P availability, which may suppress the proliferation or activity of *pqqC*-harboring microorganisms because P-cycling microbes may become less active or remain dormant when available P is sufficient [13,26,27]. Conversely, Zhang et al. [28] suggested that higher P availability in soils supported more nutrients for bacteria, boosting their growth, reproduction, and diversity. In addition, continuous cultivation of a single perennial crop has been reported to signifi-

cantly reduce bacterial diversity and alter soil microbial community composition [1,29,30]. In contrast, Zhang et al. [31] reported that grape cultivation in decertified areas improved soil properties and enhanced microbial diversity. Song et al. [32] previously reported that soil bacterial community diversity was markedly elevated in vineyard soils with 15 and 20 years of cultivation duration. This phenomenon may be attributed to the annual input of natural litter from grape branches and leaves, which effectively facilitates the enhancement of soil microbial diversity. Similarly, Li et al. [33] found that bacterial richness and diversity increased during the transition from desert land to 5-year-old cotton fields and then remained relatively stable after five years of cultivation. The inconsistencies among previous studies may be attributed to differences in soil type, crop species, fertilization regime, regional climate, management practices, and cultivation history.

The mobilization and availability of soil Pi in long-term orchard systems are jointly regulated by soil physicochemical properties and microbial communities. For example, in pomelo orchards, long-term cultivation for more than 10 years led to the accumulation of different soil P fractions, particularly readily soluble P, Al-P, and Fe-P, thereby increasing P bioavailability as well as the potential environmental risk in acidic soils [10]. In citrus orchards, Wang et al. [20] reported that the U group (representing 25 years of cultivation with soil pH < 4.8) contained significantly higher Al-bound P (Al-P) than the S group (representing 15 years of cultivation with pH 4.8–5.4) and the O group (representing 5 years of cultivation with pH 5.5–6.8), which contributed to enhanced soil P bioavailability. This pattern may be partly explained by the fact that decreasing soil pH can weaken cation-oxygen bonds and promote the release of metal cations, thereby reducing phosphate adsorption and facilitating the dissolution and transformation of inorganic phosphates from poorly soluble forms into more available fractions [34,35]. Soil pH and soil organic matter (SOM) can also indirectly influence the transformation of Al-P and Fe-bound P (Fe-P), as well as the accumulation of available P, by regulating the composition of *pqqC*-harboring bacterial communities under long-term nitrogen fertilization, such as a 10-year N addition regime [8]. Similarly, with increasing grapevine cultivation duration, including 0.5, 4, 16, and 22 years, the concentrations and relative proportions of labile and moderately labile Pi increased, while stable Pi showed a decline. These changes were strongly linked to soil pH, SOC content, *pqqC* gene abundance, and the diversity of *pqqC*-containing bacterial communities [36].

Collectively, although increasing attention has been paid to the dynamics of soil P fractions and P-cycling microorganisms in orchards with different planting years, less is known about how long-term vineyard cultivation is associated with changes in Pi fractions and *pqqC*-harboring bacteria, when compared with adjacent uncultivated soils. Such long-term cultivation is commonly accompanied by intensive fertilizer inputs, repeated return of plant residues, continuous monoculture, and progressive soil acidification. These factors may interactively regulate *pqqC*-harboring bacterial communities and soil Pi availability. In particular, elevated Pi availability may reduce the selective pressure for maintaining high *pqqC* gene abundance, whereas shifts in soil pH and substrate availability may simultaneously alter the diversity and community composition of *pqqC*-harboring bacteria. Therefore, elucidating the linkages between edaphic conditions, *pqqC*-harboring communities, and inorganic P pools is essential for improving our understanding of P mobilization mechanisms in long-term vineyard soils. In this study, soils from 20-year-old grape orchards were compared with adjacent uncultivated background soils in Penglai District, Yantai, China. The objectives were to (1) evaluate the effects of long-term grape cultivation on soil properties and Pi fractions; (2) investigate the abundance, diversity and composition of *pqqC*-harboring bacterial communities; and (3) identify the relationships linking soil environmental characteristics, *pqqC*-containing microbial assemblages, and soil Pi spe-

ciation. We hypothesized that (1) long-term grape cultivation would decrease soil pH and drive the redistribution of Pi toward labile and moderately labile fractions; (2) soil acidification, together with increased Pi availability, would decrease *pqqC* gene abundance while altering the diversity and composition of *pqqC*-harboring bacterial communities; and (3) distribution of Pi fractions would covary strongly with soil pH and the characteristics of these bacterial communities.

2. Materials and Methods

2.1. Study Area and Soil Sampling

Field investigations were carried out in Penglai District, Yantai City, Shandong Province, China, at approximately 37°45' N and 120°50' E. The region is characterized by a warm-temperate monsoon climate, with a mean annual temperature of 12.9 °C and an annual precipitation of approximately 661 mm. The soils are classified as brown soils, corresponding to Udic Luvisols according to the World Reference Base (WRB), and have a sandy loam texture. Four grape orchards cultivated for 20 years (Y20) and four adjacent uncultivated background soils (CK) were selected for soil sampling in September 2022. The CK sites, wasteland near the grape orchards with no history of crop cultivation or fertilizer application, were selected at least 10 m from the orchard boundary to minimize edge effects and fertilizer drift. In the vineyard, water-soluble fertilizer was applied 3–4 times annually through the drip irrigation system at a rate of 75 kg ha^{−1} per application. After harvest each year, compound fertilizer was applied at 750 kg ha^{−1}. Secondary and micronutrient fertilizers supplying calcium, magnesium, manganese, zinc, and other trace elements were also applied annually at a total rate of 375–600 kg ha^{−1}. Irrigation was supplied by drip irrigation throughout the growing season.

The topsoil (0–20 cm) was sampled because it harbors most microbial activity and is a key zone for anthropogenically influenced soil distribution, warranting a depth of 0–20 cm. However, as orchard cropping years increase, subsurface (20–40 cm) processes could affect P redistribution [10], suggesting a focus on P transformation in deeper soil layers. In each grape orchard and CK site, five soil cores were obtained randomly from the 0–20 cm layer using a soil auger with a 5 cm diameter, with at least 6 m spacing between cores. The five cores were thoroughly mixed to form one composite sample and then passed through a 2 mm sieve. Each fresh soil sample was divided into several portions for different analyses. One portion was immediately frozen at −80 °C and used for DNA extraction. A second portion was stored at 4 °C for the determination of microbial biomass carbon (MBC). The remaining material was air-dried and used to analyze P fractions, soil pH and SOC.

2.2. Soil Properties Analysis

Soil pH was measured using a digital pH meter at a soil-to-water ratio of 1:2.5 *w/v*. The SOC content was determined using the K₂Cr₂O₇ oxidation method [37]. Soil MBC was quantified through the chloroform fumigation-extraction method [38]. A modified sequential extraction scheme was employed to separate soil P into fractions differing in chemical stability and potential bioavailability [39,40]. Air-dried soil (0.5 g) was extracted consecutively with an anion-exchange resin, 0.5 M NaHCO₃, 0.1 M NaOH, 1 M HCl, and 0.1 M NaOH. The fractions obtained at each stage were designated as Resin-Pi; NaHCO₃-Pi and NaHCO₃-Po; NaOH1-Pi and NaOH1-Po; HCl-Pi; and NaOH2-Pi and NaOH2-Po, respectively. The residue remaining after the final extraction was digested in an H₂SO₄-H₂O₂ mixture to quantify residual P [41]. Phosphorus in each extract was analyzed by the molybdenum blue colorimetric assay [42]. The fractions were further consolidated according to their relative biological availability [43]. The inorganic P pools comprised

labile Pi (Resin-Pi + NaHCO₃-Pi), moderately labile Pi (NaOH1-Pi), and stable Pi (HCl-Pi + NaOH₂-Pi). Organic P was divided into labile (NaHCO₃-Po), moderately labile (NaOH1-Po), and stable (NaOH₂-Po) pools.

2.3. Soil DNA Extraction and Quantification of the *pqqC* Gene

For each sample, total DNA was recovered from 0.50 g of soil using the FastDNA[®] Spin Kit for Soil (MP Biomedicals, Solon, OH, USA) following the supplier's instructions. The quantity and purity of the DNA extracts were measured with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA), and their integrity was verified on a 1% agarose gel. Absolute *pqqC* gene copy numbers were subsequently measured by qPCR with the previously published primer set, consisting of Fw (5'-AACCGCTTCTACTACCAG-3') and Rv (5'-GCGAACAGCTCGGTCAG-3') [44]. Reactions were prepared in a final volume of 10 µL by combining 5 µL of 2× ChamQ SYBR Color qPCR Master Mix, 0.4 µL of each primer, 1 µL of extracted DNA, 0.2 µL of 50× ROX Reference Dye 1, and 3 µL of ddH₂O. The qPCR conditions were 95 °C for 3 min, followed by 40 amplification cycles comprising 5 s at 95 °C, 30 s at 58 °C, and 1 min at 72 °C. Quantification standards were prepared through tenfold serial dilution of recombinant plasmids harboring the target *pqqC* fragment, and the resulting standard curve was used to calculate gene copy numbers. The copy number of the *pqqC* gene was expressed as copies per gram of dry soil. The amplification efficiency, the R² value of the standard curve, and the melting curve analysis were presented in the Supplementary Materials.

2.4. High-Throughput Sequencing and Sequence Processing

The *pqqC* gene amplicons were generated from DNA extracted from each soil sample using the primer set described above. PCR amplification was initiated with a denaturation step at 95 °C for 3 min, followed by 35 cycles consisting of denaturation at 95 °C for 30 s, primer annealing at 55 °C for 30 s, and extension at 72 °C for 45 s. A final extension was then performed at 72 °C for 10 min. After purification, the resulting amplicons were converted into sequencing libraries using the NEXTFLEX[®] Rapid DNA-Seq Kit (San Jose, CA, USA). Library preparation involved adapter ligation, removal of adapter dimers using magnetic beads, PCR-based enrichment, and a subsequent bead-purification step. The completed libraries were sequenced on an Illumina MiSeq platform by Majorbio (Shanghai, China). Paired-end reads generated from sequencing were assembled with FLASH v1.2.11, and the merged sequences were further subjected to quality control and filtering using QIIME v1.9.1 [45]. High-quality sequences were clustered into operational taxonomic units (OTUs) at 97% sequence similarity using UPARSE 11.0 [44,46]. Taxonomy assignment to sequences was performed using NT (nt_v20210917) database at a confidence threshold of 0.8. After rarefying all samples to the same sequencing depth based on the sample with the lowest number of sequences, α-diversity indices, including Chao1 richness and Shannon diversity, were calculated using QIIME 1.9.1. A total of 426,840 high-quality filtered sequences with a range of 38,904–67,079 in the *pqqC*-harbouring bacterial community were produced. The sequence data were deposited in NCBI (PRJNA1492556).

2.5. Statistical Analysis

Differences in measured variables between Y20 and CK treatments were assessed using an independent-samples *t*-test ($p < 0.05$). To compare *pqqC*-harboring community composition across treatments, a principal coordinate analysis (PCoA) with Bray–Curtis distance was performed, and the significance of community separation was tested using Adonis analysis. The sample scores along the first PCoA axis were used as indicators of *pqqC*-harboring bacterial community structure in subsequent analyses. Biomarkers associated with different treatments were identified using linear discriminant analysis

(LDA) effect size (LEfSe) based on the Kruskal–Wallis test, with a logarithmic LDA > 3.0 and $p < 0.05$. The linkages of *pqqC* gene community structure with different soil factors were explored by canonical correlation analysis (CCA). A correlation network between *pqqC*-harboring genera among the top 50 genera in relative abundance and soil P fractions was constructed based on Spearman's correlation analysis. Only significant and strong correlations $p < 0.05$ and $|\text{coefficient}| > 0.8$ were retained and visualized in the network. Betweenness centrality was calculated to evaluate the topological positions and potential roles of nodes within the network [11,47,48] (Table A1). Pearson correlation analysis was performed to examine the relationships between Pi fractions and soil properties, including soil pH, SOC, and MBC, as well as microbial properties, including *pqqC* gene abundance, Chao1 index, Shannon index, and *pqqC*-harboring bacterial community composition.

3. Results

3.1. Soil Characteristics and Distribution of P Fractions

The soil pH was in the range of 6.88–7.85 (average value: 7.55; standard error: 0.23) under CK treatment and decreased significantly to 6.39–6.64 (average value: 6.57; standard error: 0.06) under Y20 treatment (Figure 1A, $p < 0.05$). The contents of SOC and MBC were in the range of 7.73–12.94 g kg^{−1} (CK: average value 10.20 and standard error 0.90; Y20: average value 11.54 and standard error 0.67) and 85.00–140.08 mg kg^{−1} (CK: average value 106.27 and standard error 7.56; Y20: average value 120.30 and standard error 8.89), respectively, with no significant difference between the two treatments (Figure 1B,C, $p > 0.05$). The average values of soil resin-Pi, NaHCO₃-Pi, NaHCO₃-Po and NaOH1-Pi in the Y20 treatment were 24.37, 40.08, 20.59 and 78.37 mg kg^{−1}, respectively, significantly higher than those observed in the CK treatment (Figure 2A, $p < 0.05$). After classification for soil Pi fractions, the average labile-Pi and moderately labile-Pi contents under CK treatment were 28.28 and 35.88 mg kg^{−1}, respectively, and increased remarkably to 64.45 and 78.37 mg kg^{−1}, respectively, under Y20 treatment (Figure 2B, $p < 0.05$).

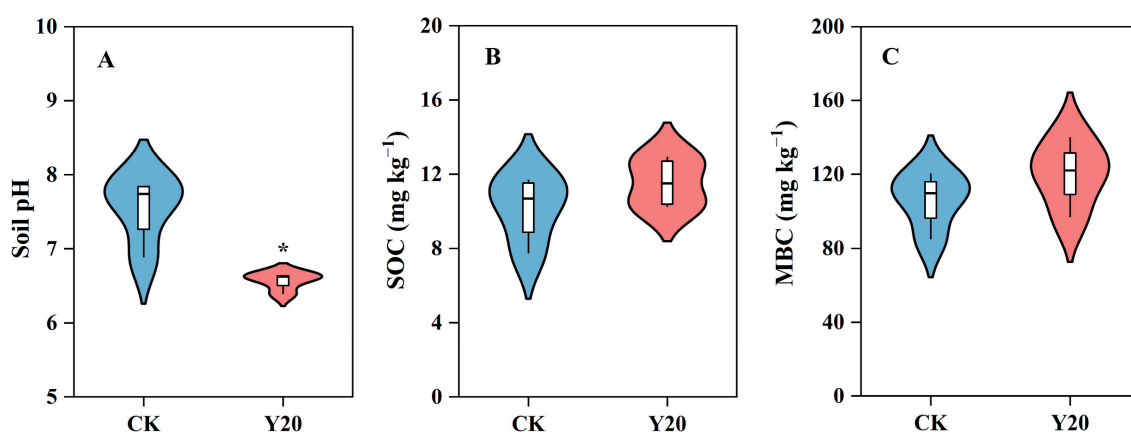


Figure 1. Changes in soil pH (A), soil organic carbon (B) and microbial biomass carbon (C) in uncultivated soils and grape orchard soils. SOC: soil organic carbon; MBC: microbial biomass carbon; CK: uncultivated soils; Y20: grape orchards cultivated for 20 years. * indicate significant differences between CK and Y20 ($p < 0.05$).

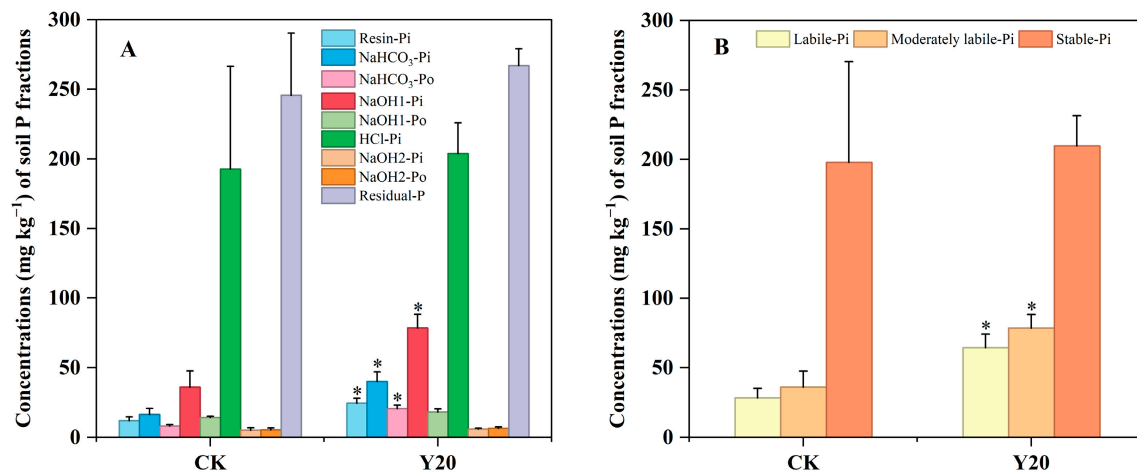


Figure 2. Concentrations of individual P fraction (A) and their distribution among different lability pools (B) in uncultivated soils and grape orchard soils. Values are expressed as means, with error bars showing standard errors. Pi: inorganic P; Po: organic P; CK: uncultivated soils; Y20: grape orchards cultivated for 20 years. * indicate significant differences between CK and Y20 ($p < 0.05$).

3.2. *pqqC* Gene Abundance and Diversity of the *pqqC*-Harboring Bacterial Community

A significant difference in *pqqC* gene abundance was observed between the two treatments, with Y20 exhibiting a lower value than CK (Figure 3A, $p < 0.05$). Chao1 and Shannon indices were used to estimate the α -diversity of *pqqC*-harboring bacterial community. Compared with CK treatment, the Chao1 index was similar between the two treatments, whereas the Shannon index was significantly higher under Y20 treatment (Figure 3B,C, $p < 0.05$). The PCoA coupled with Adonis revealed significant difference in the composition of *pqqC*-harboring bacterial communities between the two treatments (Figure 3D, $p < 0.05$).

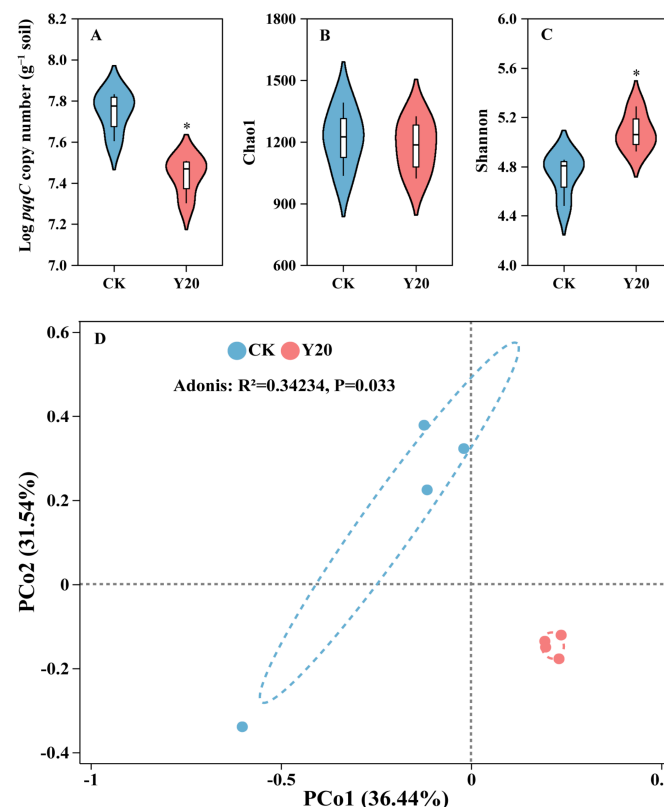


Figure 3. The abundance (A), α -diversity (B,C) and β -diversity (D) of *pqqC*-harboring bacterial communities in uncultivated soils and grape orchard soils. α -diversity estimated by Chao1 and

Shannon. Principle coordinate analysis (PCoA) plot for the *pqqC* gene community structure using Bray–Curtis dissimilarities. The ellipses indicate 95% confidence intervals for the respective treatments. Differences in community composition among treatments were evaluated using Adonis. CK: uncultivated soils; Y20: grape orchards cultivated for 20 years. * indicate significant differences between CK and Y20 ($p < 0.05$).

3.3. Taxonomic Composition and Potential Biomarkers of *pqqC*-Harboring Community

Actinobacteria and *Proteobacteria* were the predominant phyla detected in all samples (Figure 4A). The responses of various phyla to long-term orchard plantation were similar, with none showing a significant difference between Y20 and CK (Figure 4A, $p < 0.05$). Among genera, the six most abundant *pqqC*-harboring genera were *Rubrobacter*, *Variovorax*, *Geodermatophilus*, *Bradyrhizobium*, *Streptomyces* and *Pseudomonas* (Figure 4B). Compared with CK, Y20 resulted in significantly greater relative abundances of *Variovorax* and *Geodermatophilus* (Figure 4B, $p < 0.05$).

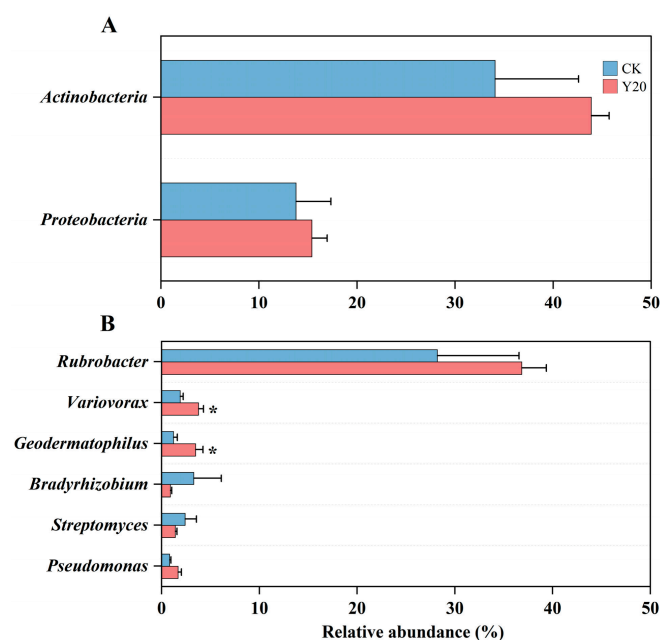


Figure 4. The main phyla (A) and genera (B) containing *pqqC* gene in uncultivated soils and grape orchard soils. Values are expressed as means, with error bars showing standard errors. CK: uncultivated soils; Y20: grape orchards cultivated for 20 years. * indicate significant differences between CK and Y20 ($p < 0.05$).

The LEfSe analysis further distinguished the two treatments based on their characteristic microbial taxa, demonstrating marked differences in the taxonomic profiles of *pqqC*-harboring bacteria (Figure 5A,B, Kruskal–Wallis test with $p < 0.05$ and log LDA > 3.0). A total of 21 bacterial taxa differed significantly, consisting of 4 taxa for CK and 17 taxa for Y20 (Figure 5A,B, $p < 0.05$). The CK-associated biomarkers were mainly from *Proteobacteria*, while the Y20-associated biomarkers predominantly originated from *Proteobacteria* and *Actinobacteria* (Figure 5A).

The CCA result revealed that the first two axes explained 25.42% and 21.96% of the total variation in *pqqC*-harboring bacterial community composition, respectively (Figure 6), while axis 3 (CCA3) accounted for 10.75% (Figure A1). Soil pH and labile-Pi were key drivers shaping the *pqqC*-harboring community composition (Table 1, $p < 0.05$).

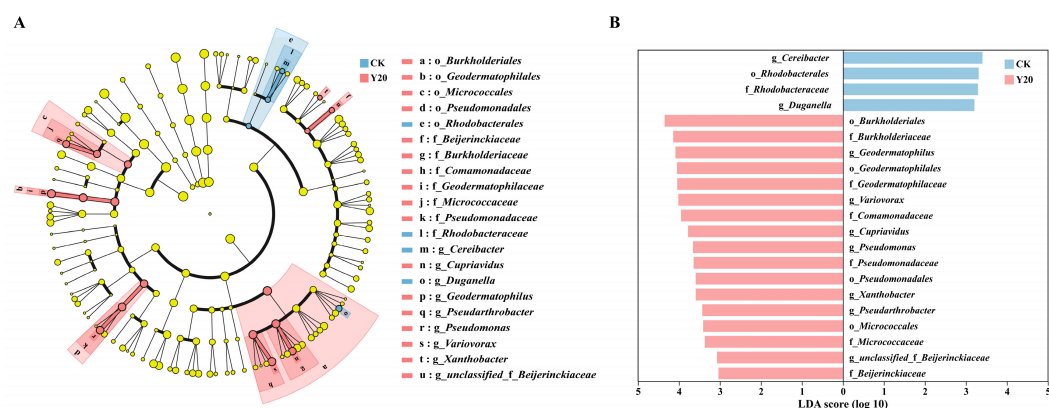


Figure 5. Phylogenetic dendrogram (A) and linear discriminant analysis (LDA) scores (B) of biomarkers between uncultivated soils and grape orchard soils. Circles in the cladogram (A) from inside to outside indicate bacterial taxonomic levels from phylum to genus. Blue and red dots represent the biomarker bacteria significantly varying in abundance among uncultivated soils and grape orchard soils, respectively. The LDA scores (B) are shown as horizontal bars for the biomarker bacteria with an LDA score > 3.0 , $p < 0.05$. The lowercase letters represent the taxonomic level of the biomarker bacteria as: o, order; f, family; g, genus. CK: uncultivated soils; Y20: grape orchards cultivated for 20 years.

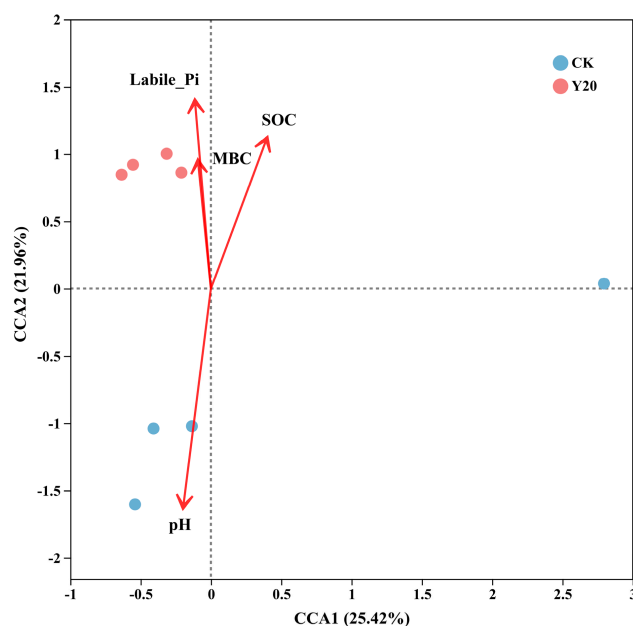


Figure 6. Canonical correlation analysis (CCA) assessing the relationship between *pqqC* gene community structure and soil properties. SOC: soil organic carbon; MBC: microbial biomass carbon; Pi: inorganic P; CK: uncultivated soils; Y20: grape orchards cultivated for 20 years.

Table 1. Statistical results of canonical correlation analysis (CCA) between *pqqC* gene community structure and explanatory environmental parameters.

Explanatory Environmental Parameters	CCA1	CCA2	r^2	p -Value
pH	−0.10702	−0.99426	0.96166	0.004
Labile_Pi	−0.06947	0.99758	0.69887	0.035
SOC	0.29924	0.95418	0.49247	0.229
MBC	−0.08283	0.99656	0.31889	0.428

MBC: microbial biomass carbon; SOC: soil organic C; Pi: inorganic phosphorus.

3.4. Relationships Linking Soil Environmental Characteristics, *pqqC*-Containing Microbial Assemblages, and Soil P Speciation

In the network, 30 highly significant Spearman correlations were observed between soil P fractions and *pqqC*-harboring microbial genera (top 50 in relative abundance) (Figure 7, $r > 0.8$ and $p < 0.05$). Significant associations were observed between soil Pi fractions, including resin-Pi, NaHCO_3 -Pi, NaOH_1 -Pi, HCl -Pi, and NaOH_2 -Pi, and *pqqC*-harboring genera (Figure 7). Similar significant relationships were also detected between soil Po fractions (NaOH_1 -Po and NaOH_2 -Po) and these genera (Figure 7). Network analysis based on betweenness centrality identified *Azospira* as the most influential taxon, with *Variibacter*, *Burkholderia*, and *Bosea* also occupying important positions (Table A1). Results of Pearson's correlation showed that the contents of resin-Pi, NaHCO_3 -Pi, NaOH_1 -Pi, labile-Pi and moderately labile-Pi were significantly and negatively correlated with soil pH and *pqqC* gene abundance, while the NaHCO_3 -Pi content was significantly and positively related to Shannon index (Figure 8, $p < 0.05$).

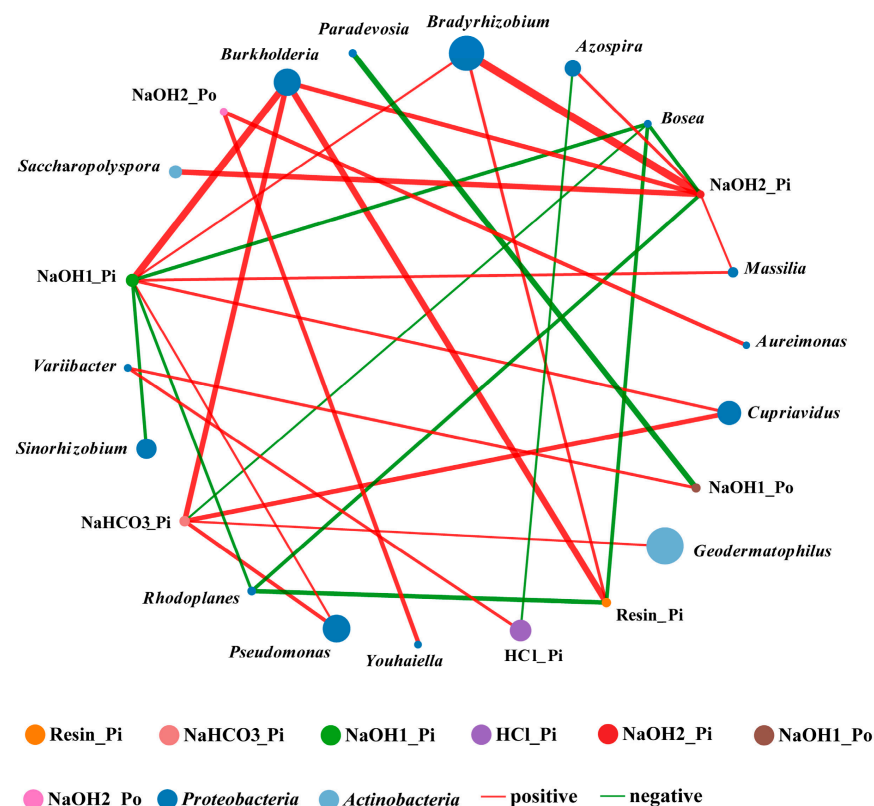


Figure 7. Spearman correlation network showing associations between the 50 most abundant *pqqC*-harboring genera and soil P fractions. Connections are shown only for statistically significant, strong correlations ($r > 0.8$, $p < 0.05$). Edge colors denote correlation direction, with red indicating positive and green indicating negative associations; line width increases with correlation magnitude. Genus nodes are colored according to their phylum-level classification, while node size represents the relative abundance of each *pqqC*-harboring genus or the corresponding concentration of a soil P fraction. Pi and Po denote inorganic P and organic P, respectively.

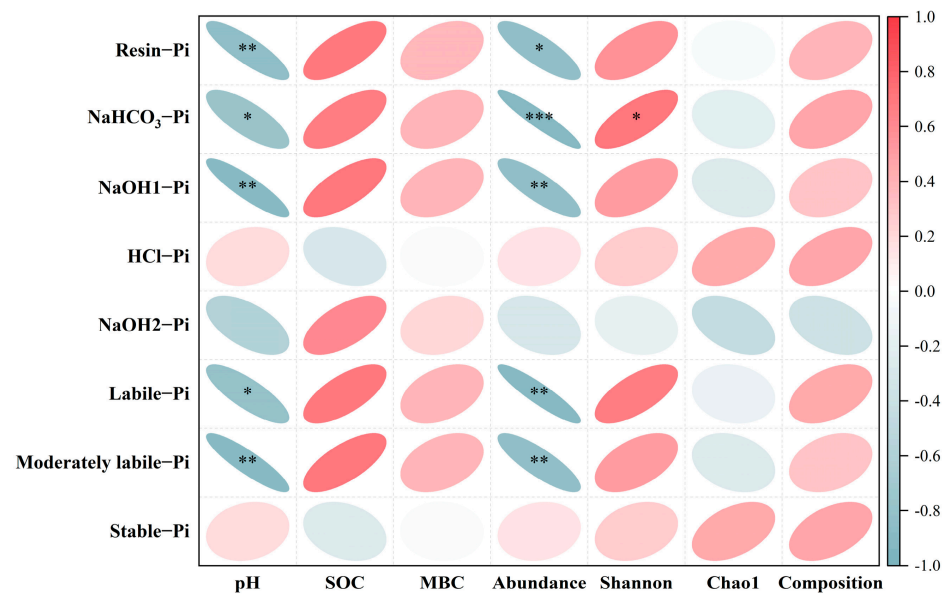


Figure 8. Correlation heatmap of soil Pi fractions and environmental properties under uncultivated soils and grape orchard soils. Colors indicate the direction of the correlation. MBC: microbial biomass carbon; SOC: soil organic C; Pi: inorganic P. * Correlation is significant at the 0.05 level. ** Correlation is significant at the 0.01 level. *** Correlation is significant at the 0.001 level.

4. Discussion

4.1. Soil Acidification Under Long-Term Grape Cultivation Promoted the Redistribution of Pi into More Available Pools

Long-term grape cultivation for 20 years resulted in a pronounced decrease in soil pH. This decline was likely associated with soil acidification induced by excessive and continuous fertilizer inputs, which is in agreement with previous studies on long-term orchard systems [5,16,49]. In contrast to soil pH, SOC and MBC showed only minor changes. The relative stability of SOC may be partly explained by the repeated incorporation of easily decomposable organic materials, such as vine residues and leaf litter, which can accelerate SOC turnover via priming effects and consequently limit net soil C accumulation [50,51]. Furthermore, although soil acidification may facilitate the accumulation of stabilized soil C, it is often accompanied by substantial depletion of labile C pools and reduced plant-derived C inputs [16]. The increase in stabilized C may therefore be insufficient to compensate for the combined effects of decreased plant inputs and extensive labile C loss, ultimately resulting in nonsignificant changes in SOC under acidified conditions [16]. In addition, MBC displayed a distribution pattern similar to that of SOC, suggesting that the size of the soil microbial biomass pool is generally closely associated with organic matter inputs [52–55]. Future studies should include measurements of specific C fractions, such as labile C or stabilized C pools, to better understand the effects of vineyard cultivation on soil C cycling.

The present study demonstrated that long-term orchard cultivation substantially altered the distribution of soil P fractions. In particular, soils from the 20-year-old orchard contained greater concentrations of labile Pi, including resin-Pi and NaHCO₃-Pi, as well as moderately labile Pi, mainly represented by NaOH₁-Pi. This result suggested that prolonged orchard cultivation promoted the redistribution of inorganic P toward more exchangeable and biologically available pools. This shift was likely linked to the drop in soil pH resulting from continued fertilizer application. Soil pH is widely regarded as a key regulator of soil P chemistry and has been identified as an important factor governing the transformation and turnover of Pi fractions [56,57]. In the 20-year-old orchard,

soil acidification may have weakened cation-oxygen bonds and enhanced cation release, thereby reducing phosphate adsorption and facilitating the dissolution and transformation of inorganic phosphates from poorly soluble forms into more available forms [34,35]. This explanation was further supported by the significant negative correlations observed between soil pH and both labile and moderately labile Pi fractions (Figure 8). In addition, organic P compounds contain P atoms covalently linked to C via phosphoester bonds, such as those in phosphomonoesters and phosphodiester [58]. Therefore, microbial C demand may stimulate the mineralization of soil organic P into inorganic P [59–61]. Furthermore, previous studies have shown that C source addition can markedly enhance microbial Po synthesis compared with treatments without C amendment [62,63]. Thus, the increased NaHCO₃-Po content in the 20-year-old orchard may be partly attributable to long-term exogenous inputs of fertilizers and organic materials. Notably, HCl-Pi and residual-P constituted substantial proportions of total soil P, and neither fraction differed significantly between CK and Y20 treatments. This pattern suggested that these pools were relatively insensitive to long-term grape cultivation, likely because they were associated with mineral-bound or recalcitrant P forms [43]. Ecologically, these fractions may serve as long-term P reservoirs and contribute to the P storage capacity of soils. Overall, the observed redistribution of soil Pi fractions may help relieve plant P limitation and improve soil P availability, while simultaneously enhancing the long-term retention of P in soil. However, the accumulation of more labile P pools may also increase the risk of P loss from the orchard soil to surrounding aquatic environments.

4.2. Long-Term Grape Cultivation Reshaped the Abundance, Diversity, and Composition of *pqqC*-Harboring Bacterial Communities

The reduced abundance of the *pqqC* gene in the 20-year-old orchard was consistent with previous studies showing that long-term continuous monoculture can decrease soil microbial abundance and diversity and alter microbial community composition [2,30,64]. However, contrasting results have also been reported. For example, based on a 28-year field experiment, Qin et al. [26] found that the abundance of the *pqqC* gene was higher under long-term mineral fertilizer application than under no-fertilizer treatment. This was attributed to the low P content in unfertilized soils, which may suppress the activity or gene expression of inorganic phosphate-solubilizing bacteria, thereby resulting in lower *pqqC* gene abundance. Previous studies have also indicated that long-term vineyard reclamation may enhance soil bacterial diversity. For instance, Song et al. [32] documented a notable rise in soil bacterial community diversity after 15 and 20 years of vineyard reclamation, probably because annual inputs of grape litter provided additional substrates for soil microorganisms. Similarly, Zhang et al. [28] suggested that higher P availability in soils supported more nutrients for bacteria, boosting their growth, reproduction, and diversity. In line with these findings, our results showed an increasing trend in the community diversity of *pqqC*-harboring bacteria, as indicated by the Shannon index, in the 20-year-old orchard. This suggested that reduced bioavailable P stress, together with annual plant residue inputs, may have stimulated certain rare or specialized taxa carrying the phosphate-mobilizing *pqqC* gene, such as members of *Mycobacteriaceae* [65]. Nevertheless, numerous studies have shown that extensive chemical fertilization can reduce the abundance of P-cycling genes responsible for P dissolution, such as *pqqC*, as well as genes associated with P uptake and transport. Such fertilization practices may also modify the diversity and community structure of soil P-utilizing microorganisms, mainly through soil acidification and associated changes in soil nutrient conditions [21–24]. The observed inconsistencies across studies can partly be attributed to variations in soil type, plant species, climate, and management practices. Therefore, the pattern observed in the 20-year-old orchard suggests that long-term grape cultivation did not simply suppress *pqqC*-harboring bacteria. Instead,

it reshaped their abundance, diversity, and taxonomic composition in different directions, probably through the combined effects of decreased soil pH, increased labile Pi availability, and continuous annual litter inputs.

The β -diversity analysis revealed a substantial difference in the composition of *pqqC* gene communities between the soil from the 20-year orchard and the background soil. Consistent with previous studies, *Actinobacteria* and *Proteobacteria* were the dominant *pqqC*-harboring bacterial phyla, whereas *Rubrobacter*, *Variovorax*, *Geodermatophilus*, *Bradyrhizobium*, *Streptomyces*, and *Pseudomonas* were the predominant genera [6,11,12,66]. These taxa have been reported to possess phosphate-solubilizing capacities and were also identified as major *pqqC*-harboring groups in the present study. Although the responses of different phyla to long-term orchard cultivation were generally similar, *Variovorax* and *Geodermatophilus* exhibited substantially higher relative abundances in the soil from the 20-year-old orchard. Moreover, LEfSe analysis showed that six of the seventeen biomarkers enriched in the 20-year-old orchard were affiliated with *Actinobacteria*. *Actinobacteria* are widely recognized as oligotrophic microorganisms because of their unique cell wall structures and their strong capacity to break down complex C substrates [67–69]. These traits may allow them to survive and function under nutrient-limited soil conditions. The dominant genus *Variovorax* is capable of degrading diverse organic compounds, such as polycyclic aromatic hydrocarbons and plant residues, and using them as sources of carbon and nutrients [66]. In addition, crop residues can release substrates, including labile and moderately labile Pi, at the residue-soil interface, thereby providing favorable microsites for phosphate-solubilizing bacteria [70]. The genus *Geodermatophilus* is renowned for its phosphate-solubilizing ability and chemotactic responses, which may facilitate its movement toward residue-derived nutrient hotspots under residue input conditions [71–74]. Therefore, the enrichment of these phosphate-solubilizing bacteria in the 20-year-old orchard was likely associated with increased P substrates, enhanced stabilized C pools, and reduced soil pH. In agreement with previous evidence that soil pH strongly influences the abundance, diversity, and composition of Pi-solubilizing bacteria [6,8,13,75], our CCA further identified soil pH as a key environmental factor structuring *pqqC*-harboring bacterial communities. Moreover, labile Pi also emerged as a key predictor of *pqqC*-harboring bacterial community composition. In P-deficient soils, P-mobilizing bacteria may adjust the composition of *pqqC*-harboring communities to enhance Pi solubilization and improve soil P availability [44]. Conversely, because labile Pi is the end product of phosphate-solubilization processes, changes in labile Pi availability may also feed back to regulate the composition of microbial populations carrying the *pqqC* gene [13].

4.3. The Distribution of Pi Fractions Was Jointly Regulated by Soil pH and *pqqC*-Harboring Bacterial Communities

The network analysis clearly showed that *pqqC*-harboring bacteria were closely associated with soil P fractions. Consistent with Abbasi et al. [47], who linked higher betweenness centrality to greater influence over network communication, our study identified *Azospira* as the top betweenness holder among *pqqC*-bearing taxa, indicating a central connector role for soil P fractions under long-term orchard cultivation. Previous evidence suggested that *pqqC*-harboring microorganisms participate in interconnected P, C, and N cycling processes. The dual capacity of *Azospira* for insoluble P mobilization and N fixation provided a potential microbial link between P and N transformations [6,76,77]. Similarly, the broad functional attributes of keystone taxa within *pqqC* networks indicated that P turnover is closely integrated with C and, particularly, N cycling [44]. Stable Po might also shape *pqqC*-harboring bacterial communities indirectly through its interactions with Pi pools, suggesting that Pi solubilization and Po mineralization were not independent processes [11]. This interpretation is supported by the highly significant relationships

observed in our study between *pqqC*-harboring genera and all Pi fractions, together with moderately labile and stable Po. These relationships suggested that *pqqC*-harboring bacteria played an important role in Pi turnover and that Pi solubilization may occur in parallel with Po mineralization. Therefore, future studies should further investigate the coupling mechanisms among P fractions, including both Pi and Po, and N cycling in orchard soils. However, a limitation of this study is the relatively small sample size, with four biological replicates per treatment. This small sample size may reduce statistical power and affect the stability of analyses that are particularly sensitive to sample size, such as correlation network analysis, LEfSe biomarker identification, and multivariate statistical approaches. Consequently, the associations and group differences identified in these analyses should be interpreted cautiously, as they may be influenced by sample-to-sample variation. Accordingly, these findings should be considered preliminary, and future studies with larger sample sizes and independent validation datasets will be necessary to confirm the observed results reported here.

Our findings further highlight the importance of both abiotic and microbial factors in regulating soil Pi mobilization. Soil pH exerted a strong influence on Pi fractions, particularly labile Pi and moderately labile Pi, as indicated by their significant negative correlations. As discussed above, this pattern may be attributed to the greater availability of protons under acidic conditions, which can promote P release through desorption processes [34,35]. In addition, the significant negative effect of *pqqC* gene abundance on soil Pi fractions may reflect both the capability of *pqqC*-containing bacteria to solubilize inorganic P and the potential feedback inhibition of *pqqC* gene expression or proliferation under rapidly increasing inorganic orthophosphate concentrations [6,13,26]. However, because this inference is based solely on gene abundance, it does not necessarily reflect actual transcriptional expression or enzymatic activity. Therefore, transcriptomic, proteomic, and enzymatic assays would be required to confirm the underlying functional mechanism. Moreover, a significant positive relationship was observed between the α -diversity of the *pqqC*-harboring bacterial community and NaHCO_3 -Pi content. This relationship may be explained by the P-solubilizing ability of these communities, as well as by reduced bioavailable P stress, which may favor certain rare or specialized taxa carrying the P-mobilizing *pqqC* gene [65]. Our results revealed that shifts in the fractions of Pi are tightly linked to soil pH, the *pqqC* gene abundance, and the diversity of *pqqC*-harboring communities during prolonged orchard cultivation.

5. Conclusions

Our results demonstrated that prolonged orchard establishment facilitated the redistribution of soil Pi toward more readily exchangeable and biologically available pools, which may increase the potential for P loss. Moreover, *pqqC* gene abundance decreased significantly, whereas its α -diversity increased significantly in the 20-year-old orchard, due to soil acidification and the low stress of bioavailable P. Meanwhile, *pqqC*-harboring community composition was significantly different between Y20 and CK, with soil pH and labile-Pi identified as key drivers. The correlation network uncovered a close link between *pqqC*-bearing bacteria and soil P fractions, with a pivotal bacterial genus (*Azospira*) acting as a key bridge. Overall, the results implied that shifts in the fractions of Pi are tightly connected to soil pH, the *pqqC* gene abundance, and the diversity of *pqqC*-harboring community under long-term orchard cultivation in the study area. However, because this study was based on a paired observational design and a limited number of samples, these relationships should be interpreted as exploratory rather than causal. Future studies combining long-term monitoring, controlled experiments, and functional assays are needed to

clarify the mechanisms linking vineyard management, soil acidification, P transformation, and *pqqC*-harboring bacterial communities.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/agriculture16151573/s1>. Table S1. qPCR amplification efficiency for the *pqqC* standard. Figure S1. Melting curve analysis of qPCR for the *pqqC* gene. Figure S2. Rarefaction curves confirming sufficient sequencing depth to capture the true diversity of *pqqC* community.

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Abbreviations

The following abbreviations are used in this manuscript:

Po	Organic phosphorus
PQQ	Pyrroloquinoline quinone
SOC	Soil organic carbon
MBC	Microbial biomass carbon
Pi	Inorganic phosphorus
N	Nitrogen
P	Phosphorus
Y20	Grape orchards cultivated for 20 years.
CK	Uncultivated soils
PSB	Phosphate-solubilizing bacteria
Al-P	Al-bound P
Fe-P	Fe-bound P
qPCR	Quantitative PCR
OTUs	Operational taxonomic units
PCoA	Principal coordinate analysis
LDA	Linear discriminant analysis
LEfSe	Linear discriminant analysis effect size
CCA	Canonical correlation analysis
SOM	Soil organic matter

Appendix A

Table A1. Betweenness centrality of members in the correlation network.

Node_Name	Betweenness_Centrality
NaOH2_Pi	0.3956
<i>g_Azospira</i>	0.2667
HCl_Pi	0.2143
NaOH1_Pi	0.2123
<i>g_Variibacter</i>	0.1524
NaHCO ₃ _Pi	0.1163
<i>g_Burkholderi</i>	0.1006
<i>g_Bosea</i>	0.1006
NaOH1_Po	0.081
<i>g_Bradyrhizob</i>	0.0364
<i>g_Rhodoplanes</i>	0.0364
<i>g_Massilia</i>	0.0229
Resin_Pi	0.0139
<i>g_Pseudomonas</i>	0.0087
<i>g_Cupriavidus</i>	0.0087
NaOH2_Po	0.0048
<i>g_Geodermatop</i>	0
<i>g_Sinorhizobi</i>	0
<i>g_Saccharopol</i>	0
<i>g_Paradevosia</i>	0
<i>g_Youhaiella</i>	0
<i>g_Aureimonas</i>	0

Pi: inorganic P; Po: organic P.

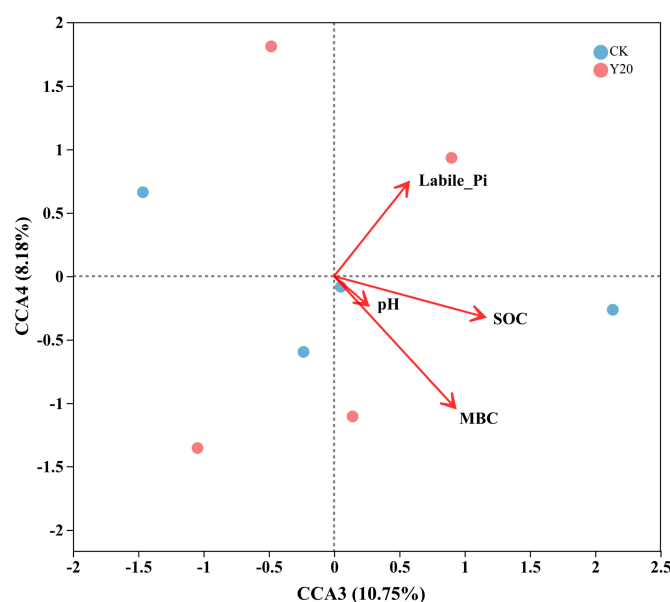


Figure A1. The variance in the *pqqC*-harboring bacterial community composition explained by Axis 3 and Axis 4 in canonical correlation analysis (CCA). MBC: microbial biomass carbon; SOC: soil organic C; Pi: inorganic phosphorus; CK: uncultivated soils; Y20: grape orchards cultivated for 20 years.

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